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14. ABSTRACT

The focus of this proposal is Neuropilin-2 (NRP2), a VEGF receptor that is not expressed in normal prostate but is expressed in prostate cancer and correlates with Gleason grade. We demonstrated that PTEN deletion induces NRP2 expression and propose that NRP2 contributes to the function of prostate cancer stem cells and tumor formation. We also discovered that NRP2 facilitates the expression of Bmi-1, a transcriptional repressor, and that NRP2 suppresses the IGF-1 receptor (IGF-1R) by a mechanism that involves transcriptional repression by Bmi-1. We have obtained preliminary evidence that targeting NRP2 directly on tumor cells in combination with IGF-1R inhibition is effective treating aggressive prostate carcinoma and pursuing this possibility more rigorously. The role of VEGF/NRP2 signaling in the function of prostate cancer stem cells and tumor initiation is also being investigated.

15. SUBJECT TERMS

Prostate cancer, VEGF, Neuropilin, IGF-1 receptor, PTEN, stem cells, therapy

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INTRODUCTION: This study is based on the premise that prostate carcinoma (PCa) cells express receptors for VEGF and that these receptors contribute to tumor initiation. The focus is on Neuropilin-2 (NRP2), a VEGF receptor that is not expressed in normal prostate but is expressed in PCa and correlates with Gleason grade. It is proposed that PTEN deletion induces NRP2 expression and that NRP2 contributes to PCa formation. The role of VEGF/NRP2 signaling in prostate tumorigenesis can be explained by the discovery that NRP2 facilitates the expression of Bmi-1, a transcriptional repressor that has a critical role in the function of PCa stem cells (1). We hypothesize that NRP2 suppresses the IGF-1 receptor (IGF-1R) by a mechanism that involves transcriptional repression by Bmi-1 and, as a consequence, confers resistance to IGF-1R therapy of prostate carcinoma. This hypothesis is significant because several IGF-1R inhibitors are in clinical trials (2) but the mechanisms to account for patient response to these inhibitors are largely unknown. Similarly, clinical trials of the VEGF Ab bevacizumab have been disappointing for reasons that are not entirely known (3) but it is worth noting that this drug does not inhibit the VEGF/NRP2 interaction (4). For these reasons, targeting NRP2 directly on tumor cells in combination with IGF-1R inhibition should be a novel and a potentially potent approach for treating aggressive prostate carcinoma.

BODY: During the first year of this award, we have made progress on the following tasks:

Task 1. Establish that VEGF/NRP2 signaling contributes to the function of tumor-initiating cells and the formation of prostate carcinoma induced by PTEN deletion.

1a. Generate proposed transgenic genotypes ($NRP2^{pc-/-}$ $cPTEN^{pc-/-}$ L, $NRP2^{pc+/-}$ $cPTEN^{pc-/-}$ L and $NRP2^{pc+/+}$ $cPTEN^{pc-/-}$ L). We initiated the breeding events needed to generate the proposed transgenic genotypes.

1d. Isolate and characterize tumor initiating cells from transgenic mouse and human tumors. We have accomplished the task of isolating tumor initiating cells from transgenic mouse and human tumors, and characterizing these cells. As shown in Fig. 1A, B, we isolated SCA^+ and SCA^- cells from $PTEN^{pc-/-}$ mice and demonstrated that the ability of SCA^+ to form prostatopheres is inhibited by a NRP2-antibody. We have also isolated NRP2high and NRP2low cells from primary prostate tumors and demonstrated that the ability of the NRP2high cells to form prostatopheres is inhibited by a NRP2-antibody.

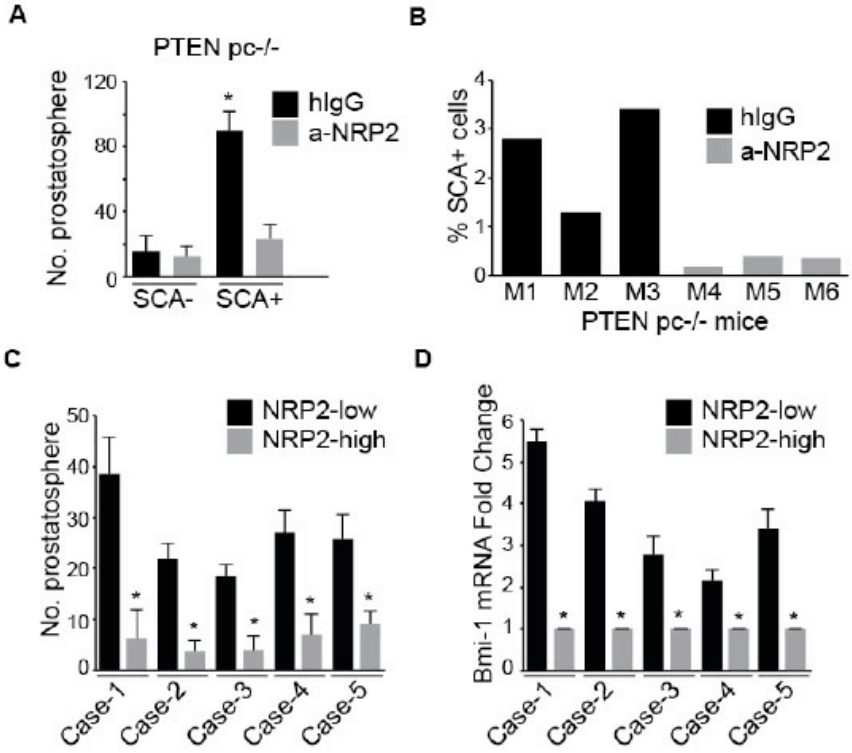

Figure 1. **A**. Primary tumors from the prostate gland of 12 week old $PTEN^{pc-/-}$ mice were isolated. Tissues were digested and LSC^+ and LSC^- populations were isolated. These populations (10,000 cells per well) were plated on low-adherent plates in the presence of either hIgG or NRP2 blocking antibody in serum-free medium. The number of prostatospheres was counted after 10 days and presented. The data are the mean of 3 independent tumors. **B**. $PTEN^{pc-/-}$ mice (6-weeks old) were injected with either hIgG or a NRP2 blocking Ab. After three weeks, tumors were harvested and the percentage of LSC^+ cells was analyzed. Graph depicts the percentage of LSC^+ cells in 6 different mice. **C**. Freshly harvested primary tumors from prostate cancer patients were digested and epithelial cells were separated into either NRP2-high or NRP2-low populations. These cells were plated to measure their ability to form prostatospheres. Tumors from five different patients were used. **D**. Freshly harvested primary tumors from prostate cancer patients were digested and epithelial cells were separated into either NRP2-high or NRP2-low populations. RNA was isolated from these cells and expression of Bmi-1 was quantified using qPCR.

Task 2. Establish that NRP2 represses the IGF-1R and assess the consequences of this regulation on the function of prostate carcinoma cells.

2b. Analyze expression of NRP2, IGF-1R and Bmi-1 in microdissected prostate tumors using qPCR and IHC. We have isolated the NRP2high and NRP2low from microdissected prosate tumors and analyzed the expression of NRP2, IGF-1R and key signaling molecules (Fig. 2A). The NRP2high population has also been transfected with IGF-1R (Fig. 2B) and the effect on prostatosphere formation assessed (Fig. 2C,D). These cells were also analyzed for expression of luminal and basal differentiation markers and CD49f (Fig. 2 E, F).

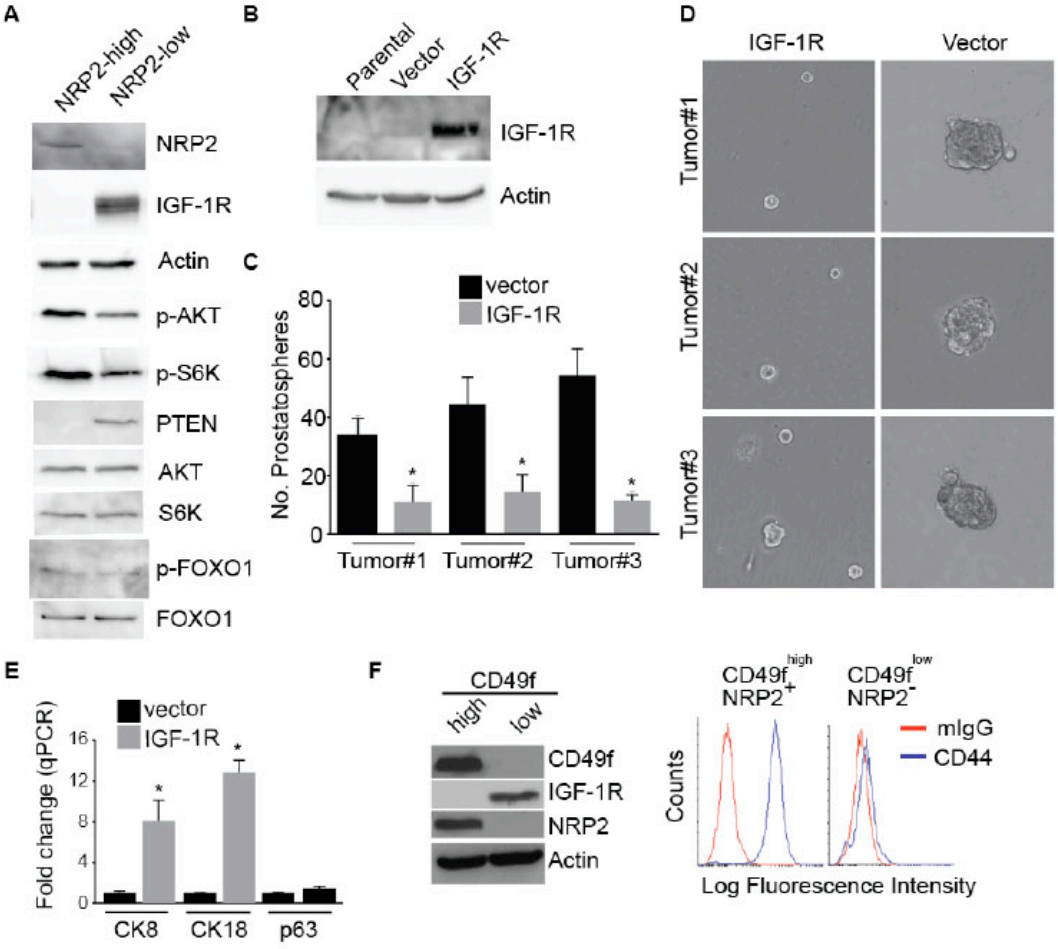

Figure 2. A. Freshly harvested primary tumors from prostate cancer patients were digested and epithelial cells were separated into either NRP2-high or NRP2-low populations. Protein extracts from these cells were used to assess the activation of various signaling proteins by immunoblotting. **B-E.** Freshly harvested primary tumors from three prostate cancer patients were digested and epithelial cells were separated into either NRP2-high or NRP2-low populations. The NRP2-high population was transfected with either empty vector or IGF-1R. Immunoblotting was done to confirm the expression of IGF-1R (**B**). These transfectants were plated to measure prostatosphere formation (**C-D**). Expression of CK8, CK18 and p63 (markers of luminal and basal differentiation) were analyzed by qPCR (**E**). **F.** Freshly harvested primary tumors from prostate cancer patients were digested and epithelial cells were separated into either CD49f-high or CD49f-low populations. Protein extracts from these cells were used to assess the expression of CD49f, IGF-1R, NRP2 and actin by immunoblotting. These cells were also used to assess expression of CD44 by FACS.

The relationship between IGF-1R and α6 integrin (CD49f) that was revealed in Fig. 2 is interesting and relevant to this aim. For this reason, we pursued this relationship in more detail using a normal prostate epithelial cell line (p69). We discovered that the IGF-1R and α6 integrin associated in these cells as evidenced by co-up (Fig. 3A) and that α6 integrin is necessary for IGF-1-mediated proliferation (Fig. 3B). Expression of NRP2 in p69 cells inhibits the association of IGF-1R with α6 integrin (Fig. 3D-G) and this effect is dependent on the VEGF binding activity of NRP2 (Fig. 3H, I).

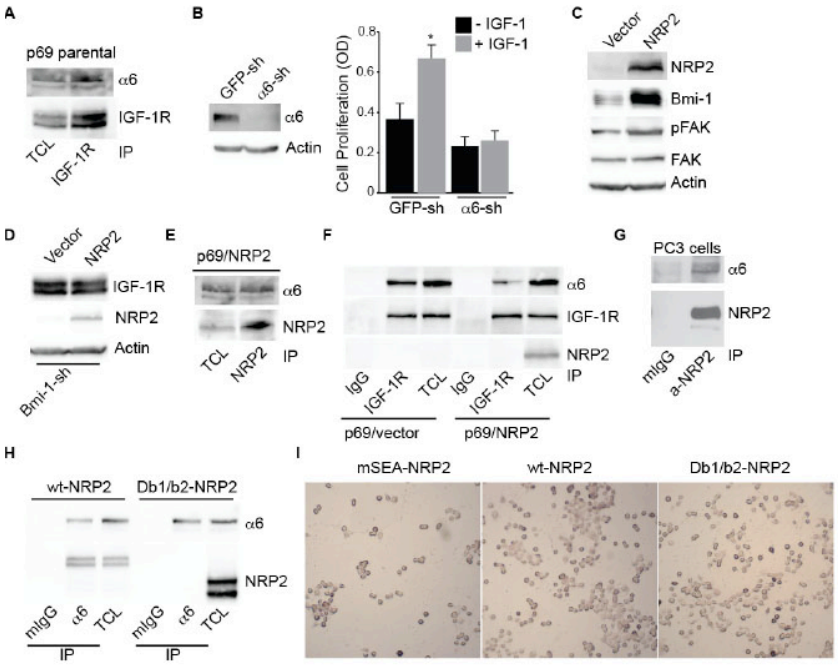

Figure 3. A. Protein extracts from the p69 normal prostate cell line were used to assess the association between α6 integrin and IGF-1R. Immunoblot shows that alpha6 integrin associates with IGF-1R. **B.** Expression of α6 integrin was downregulated using α6 shRNA as shown by immunblotting. These transfectants were used to measure the effect of IGF-1 on cell proliferation. Our data reveal that the α6 integrin is required for IGF-1 stimulated cell proliferation. **C.** Effect of NRP2 expression on NRP2, BMI-1, phospho- FAK, total FAK and actin was analyzed by immunoblotting. NRP2 expression increases Bmi-1 and phospho-FAK levels. D. Effect of NRP2 expression on IGF-1R in the absence of Bmi-1 was analyzed by immunoblotting. NRP2 expression did not change the IGF-1R levels in the absence of Bmi-1. **E.** Protein extracts from the p69 normal prostate cell line stably expressing NRP2 were used to assess the association between α6 integrin and NRP2. Immunoblot shows that α6 integrin associates with NRP2. **F.** Protein extracts from p69/vector or p69/NRP2 stable transfectants were used to assess the association between α6 integrin and IGF-1R. Immunoblot shows that NRP2 significantly inhibited the association between NRP2 and α6 integrin. **G.** Protein extracts from PC3 cells were used to assess the association between α6 integrin and NRP2. Immunoblot shows that α6 integrin associates with NRP2. **H.** p69 cells were transfected with either wt-NRP2 or mutant NRP2 (lacking b1 and b2 domain). Protein extracts from these stable transfectants were used to assess the association between α6 integrin and NRP2. Immunoblot shows that b1/b2 domain of NRP2 is required for its association with alpha6 integrins. **I.** p69 cells transfected with wt-NRP2, mSEA-NRP2 (mutated last three amino acids) or delta b1/b2 NRP2 (lack VEGF binding domain) express equally well on surface. The staining was done using AP assay reagent kit from Gene Hunter.

Task 3. Evaluate the relationship between NRP2 and IGF-1R in PCa therapy.

3c. Initiate the comparison of monotherapy using either NRP2 or IGF-1R inhibitors to dual therapy using both inhibitors combined. We have initiated these experiments using PC3 cells. The data obtained reveal that the combined used of NRP2 and IGF-1R antibodies is much more effective at reducing the growth of xenografts than either antibody alone (Fig. 4).

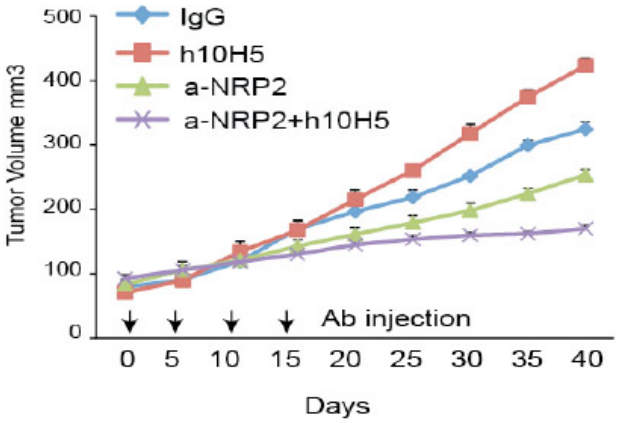

Figure 4. PC-3 cells were grown in mice until the tumor volume reached 100mm³. At this point, tumors were injected with antibodies to either IGF-1R or NRP2 or both. IgG was used as the control. There were a total of four injections (Day 0, 5, 10, and 15). Tumor volume was measured at 5 day intervals. The graph shows that the combination of NRP2 and IGF-1R antibodies is the most effective approach to inhibit the growth of the tumors.

REPORTABLE OUTCOMES:

Manuscripts:
1. Mercurio AM, Goel HL. VEGF targets the tumor cell. **Nature Reviews Cancer**, 2013, 13:871-882.

Academic Advancement:
Dr. Hira Lal Goel has been promoted from Research Assistant Professor to Assistant Professor based on his productivity from this project.

Presentations:
This work was presented in seminars at the following institutions: Boston University Medical Center (Dept. of Molecular and Cellular Biology), University of Massachusetts Amherst (Department of Animal Science), Oklahoma University Health Sciences Center (Department of Physiology).

KEY RESEARCH ACCOMPLISHMENTS:
During the past year of funding, we discovered that:

- Stem cell properties of tumor initiating cells isolated from transgenic mouse and primary human prostate tumors are dependent on NRP2.
- An inverse correlation exists between IGF-1R and NRP2 expression in prostate epithelial cells and prostate cancer that relates to differentiation. IGF-1R signaling is linked to differentiation and VEGF/NRP2 signaling promotes de-differentiation.
- The IGF-1R interacts with the α6 integrin in differentiated prostate epithelial cells and this integrin is needed for IGF signaling. Induction of NRP2 expression, which occurs in more aggressive prostate cancer, disrupts this interaction by repressing IGF-1R transcription.
- The combined use of function blocking IGF-1R and NRP2 antibodies is much more effective at reducing prostate tumor growth than either antibody alone.

CONCLUSION: The work accomplished during this year of funding provides a strong foundation for the subsequent experiments outlined in the original application. Given that we have initiated the breeding strategy outlined in Aim 1, we will be able to obtain rigorous genetic evidence implicating NRP2 in the function of prostate cancer stem cells and tumor initiation. The relationship we have uncovered between the IGF-1R and NRP2 is very interesting because it indicates that two opposing growth factor pathways exist in the prostate. IGF/IGF-1R signaling maintains a differentiated state that is associated with less aggressive tumors and fewer tumor initiating cells. Conversely, VEGF/NRP2 signaling promotes de-differentiation and is characteristic of more aggressive tumors, especially because this pathway is necessary for the function of tumor initiating cells. Continued studies on the relationship between these pathways should be insightful. We are also excited by our finding that the α6 integrin functions depend on the relative expression of either the IGF-1R or NRP2. In the presence of the IGF-1R, this integrin promotes differentiation and impedes the formation of tumor initiating cells. In marked contrast, loss of the IGF-1R enables this integrin to initiate a signaling pathway involved in the function of tumor initiating cells and that is associated with more aggressive cancers. We intend to continue our work on how this integrin contributes to prostate cancer, in the context of IGF-1R and NRP2 signaling. Finally, our function blocking antibody experiments using xenografts establish proof of principle that we can now extend to transgenic models of prostate cancer as outlined in the application.

The 'so-what' component of our research is significant. Our work has revealed that NRP2 is a valid therapeutic target for aggressive prostate cancer but that therapy must involve inhibition of the IGF-1R as well. Given that function-blocking antibodies are available for both NRP2 and IGF-1R, clinical trials could be initiated in a relatively short time. The involvement of the α6 integrin adds a new dimension that can also be exploited therapeutically.

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4. Geretti E, van Meeteren LA, Shimizu A, Dudley AC, Claesson-Welsh L, Klagsbrun M. A mutated soluble neuropilin-2 B domain antagonizes vascular endothelial growth factor bioactivity and inhibits tumor progression. Mol Cancer Res;8(8):1063-73.

APPENDIX: The Appendix contains the *Nature Reviews Cancer* publication.

SUPPORTING DATA: The supporting data are provided in the Body.

Appendix

The following item is contained in the Appendix and appended below:

Goel HL, Cheng C, Pursell B, Leav I, Lyle SR, Xi HS, Hsieh CC, Adisetiyo H, Roy-Burman P, Coleman IM, Nelson PS, Vessella RL, Davis R, Plymate SR, **Mercurio AM**. VEGF/Neuropilin-2 regulation of Bmi-1 and repression of IGF-1R define a novel mechanism of aggressive prostate cancer. **Cancer Discovery**, 2012, 2:906-921.

VEGF targets the tumour cell

Hira Lal Goel and Arthur M. Mercurio

Abstract | The function of vascular endothelial growth factor (VEGF) in cancer is not limited to angiogenesis and vascular permeability. VEGF-mediated signalling occurs in tumour cells, and this signalling contributes to key aspects of tumorigenesis, including the function of cancer stem cells and tumour initiation. In addition to VEGF receptor tyrosine kinases, the neuropilins are crucial for mediating the effects of VEGF on tumour cells, primarily because of their ability to regulate the function and the trafficking of growth factor receptors and integrins. This has important implications for our understanding of tumour biology and for the development of more effective therapeutic approaches.

Integrins
A family of more than 20 heterodimeric cell surface extracellular matrix (ECM) receptors. Integrins connect the ECM to the cytoskeleton and can transmit signalling information bidirectionally.

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Vascular endothelial growth factor (VEGF) was identified and isolated as an endothelial cell-specific mitogen that has the capacity to induce physiological and pathological angiogenesis[1,2]. In a separate context, a factor that promotes vascular hyperpermeability, vascular permeability factor, was isolated and later shown to be identical to VEGF[3,4]. This VEGF is now known as VEGFA and is a member of a larger family of growth factors that also includes VEGFB, VEGFC, VEGFD and placental growth factor (PLGF). These family members differ in their expression pattern, receptor specificity and biological functions[5]. VEGFA, which is often referred to as VEGF, has been studied more than the other members of this family and it has several distinct variants ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$ and $VEGF_{206}$). These variants occur because of alternative splicing, and they also differ in receptor specificity and function[5]. Unsurprisingly, the role of VEGFs in angiogenesis and lymphangiogenesis has dominated the VEGF research field since the initial discovery of VEGFs, and these studies have provided considerable insights into the mechanisms that underlie the complex process of angiogenesis[6]. Importantly, these studies provided the foundation for the development of anti-angiogenic therapies that target VEGF and VEGF receptors[7,8].

It has become apparent that the function of VEGF is not limited to angiogenesis and vascular permeability[9]. VEGF, for example, can affect the function of immune cells that are present in the tumour microenvironment and, consequently, it can affect the host response to tumours (see, for example, REF. 10). In addition, VEGF receptors may regulate the function of fibroblasts in the tumour stroma[11] (BOX 1; FIG. 1). One of the most interesting developments is the discovery that autocrine and paracrine VEGF signalling occur in tumour cells and that this signalling contributes to key aspects of tumorigenesis, especially the function of cancer stem cells, independently of angiogenesis (FIG. 1). Signalling downstream of VEGF in tumour cells is mediated by VEGF receptor tyrosine kinases (RTKs) and neuropilins (NRPs). The NRPs have a major role in this signalling because of their ability to interact with and to affect the function of multiple RTKs and integrins. This Review focuses on VEGF signalling in tumour cells and its implications for tumour biology and therapy.

VEGF receptors on tumour cells

VEGF RTKs and NRPs. The hypothesis that VEGF signalling contributes to the functions of tumour cells implies that tumour cells express specific VEGF receptors that mediate this signalling. The classical VEGF receptors are the RTKs VEGFR1 (also known as FLT1), VEGFR2 (also known as FLK1 and KDR) and VEGFR3 (also known as FLT4)[12]. Although the expression of these receptors was initially thought to be limited to endothelial cells, it is now known that most of these receptors are expressed by many tumour types and that their expression correlates with clinical parameters (TABLE 1). VEGFR2 is the predominant RTK that mediates VEGF signalling in endothelial cells and that drives VEGF-mediated angiogenesis[12]. Interestingly, some tumour cells express VEGFR2 and it has a prime role in mediating VEGF signalling (see, for example, REFS 13,14), but the response of other tumour cells to VEGF seems to be independent of this RTK (see, for example, REFS 15,16), which indicates that VEGF signalling in these cells is mediated by other receptors.

VEGFR1 binds to VEGF with a higher affinity than VEGFR2, but the tyrosine phosphorylation of VEGFR1 in response to VEGF is weaker[5]. This observation, together with the existence of an alternatively spliced

Key points

- Tumour cells express vascular endothelial growth factor (VEGF) receptors and respond to autocrine and paracrine VEGF signals.
- VEGF signalling in tumour cells affects tumour functions independently of angiogenesis.
- VEGF signalling in tumour cells is mediated by VEGF receptor tyrosine kinases (RTKs) and neuropilins (NRPs).
- NRPs may be at the centre of VEGF signalling because they regulate the function of RTKs and integrins that are crucial for tumour cell function.
- Autocrine VEGF signalling may be essential for tumour initiation because it regulates the size of the cancer stem cell pool and the self-renewal of cancer stem cells.
- Therapeutic approaches that aim to target NRPs and VEGF RTKs on tumour cells could be useful to promote tumour regression and to diminish the probability of tumour recurrence, especially when used in combination with VEGF-specific antibodies and other modes of therapy.

Plexins
A large family of transmembrane proteins that share homology in their extracellular domains with the MET receptor and semaphorins.

soluble form of VEGFR1, indicates that this RTK can function as a decoy receptor by sequestering VEGF from VEGFR2, thus regulating VEGFR2 signalling[17]. Nonetheless, some tumour cells express VEGFR1 in the absence of VEGFR2 and seem to use this RTK as a signalling receptor to mediate key functions[17–19]. However, the signalling mechanism of VEGFR1 remains to be elucidated. In contrast to VEGFR1 and VEGFR2, VEGFR3 does not bind VEGFA, and this RTK primarily functions in lymphangiogenesis as a receptor for VEGFC and VEGFD[5,17].

Given that some tumour cells seem to lack expression of one or more of the VEGF RTKs but respond to autocrine and paracrine VEGF signals, it can be inferred that other types of receptors mediate or contribute to VEGF signalling in these cells. In this context, during recent years, the NRPs have garnered the most attention as VEGF receptors that function in tumour initiation and progression[20,21]. The NRPs were initially identified as neuronal receptors for class 3 semaphorins, which are axon guidance factors that function in the developing nervous system[22,23]. NRPs primarily function as co-receptors because they lack an intrinsic signalling capability; for example, NRPs form a complex with specific plexins in neurons and other cell types to form functional semaphorin receptors[24,25]. The two NRPs that are expressed in vertebrates (NRP1 and NRP2) are transmembrane glycoproteins that show 44% homology at the amino acid level. They contain four distinct extracellular domains that mediate ligand binding and a short cytoplasmic domain that lacks catalytic activity[21,26,27]. Alternative splicing of NRP1 and NRP2 can produce multiple isoforms, including secreted, soluble forms and NRP2 variants that have differences in their cytoplasmic domains[28]. There is also evidence that NRPs are modified by *O*-linked glycosylation and that this glycosylation can increase ligand binding and receptor expression[11,29–31].

The crucial finding in the context of this Review is that NRPs can function as VEGF receptors and that they are expressed on tumour cells[32]. This seminal finding led to studies aiming to understand the contribution of NRPs to tumour biology. NRPs that are in a complex with specific plexins can also contribute to tumour cell function by functioning as semaphorin receptors[33] (BOX 2). Although there is some indication that plexins contribute to VEGF signalling[34], more data are needed, especially in tumour cells. The NRPs form complexes with VEGF RTKs (VEGFR1 and VEGFR2) and increase the affinity of these receptors for VEGF[35]. The NRPs can also affect the activity of many other receptors that are crucial for tumour cell function, and there is evidence that they may signal independently of other receptors. The crucial issue is whether these functions involve VEGF. In addition, the question of whether NRP1 and NRP2 in their capacity as VEGF receptors differ in their ability to affect tumour cells has not been investigated in depth, apart from the few examples that are cited below.

Regulation of VEGF signalling in tumour cells. The majority of studies that have observed VEGF signalling in tumour cells have characterized this signalling as autocrine[14,16,36–43], although paracrine signalling does occur (see, for example, REF. 44). Moreover, the existence of autocrine VEGF signalling in human tumours is supported by the observation that VEGF is expressed in tumour cells, as shown by immunohistochemical data (TABLE 1), as well as by *in situ* hybridization and by analysis of microdissected tumour cells[45,46]. This reliance on autocrine signalling might reflect the importance of VEGF in sustaining the self-sufficiency or autonomy of tumour cells — a consideration that is highly relevant to aggressive cancers and to the biology of cancer stem cells. Indeed, autocrine VEGF signalling is generally characteristic of more aggressive cancers, including poorly differentiated carcinomas[15,16,46]. More fundamentally, poorly differentiated carcinomas show an embryonic gene expression pattern and the activation of key developmental pathways[47]. There are some data that implicate such pathways in the regulation of VEGF and VEGFR expression in tumour cells. Thus, these data provide

Box 1 | **Other functions of VEGF in the tumour microenvironment**

In addition to affecting endothelial and tumour cells, vascular endothelial growth factor (VEGF) influences tumour function by targeting other cell types in the tumour microenvironment. Notably, immune cells can express VEGF receptors, and the functions of these cells can be regulated by VEGF signalling; for example, $CD4^+$ forkhead box protein P3 $(FOXP3)^+$ regulatory T cells, which suppress an antitumour immune response, express neuropilin 1 (NRP1) and are 'guided' into tumours by VEGF, which functions as a chemoattractant[10]. Ablation of NRP1 in this population of T cells increases the activation of $CD8^+$ T cells and there is a concomitant reduction in tumour growth. Macrophages in the hypoxic tumour microenvironment secrete VEGF, which contributes to the many functions of VEGF in tumours[123]. In addition to their many other functions, fibroblasts in the tumour stroma secrete VEGF. These cells express NRP1 and use it to increase fibronectin fibril assembly, which augments tumour growth; however, whether this process involves VEGF is not known[11].

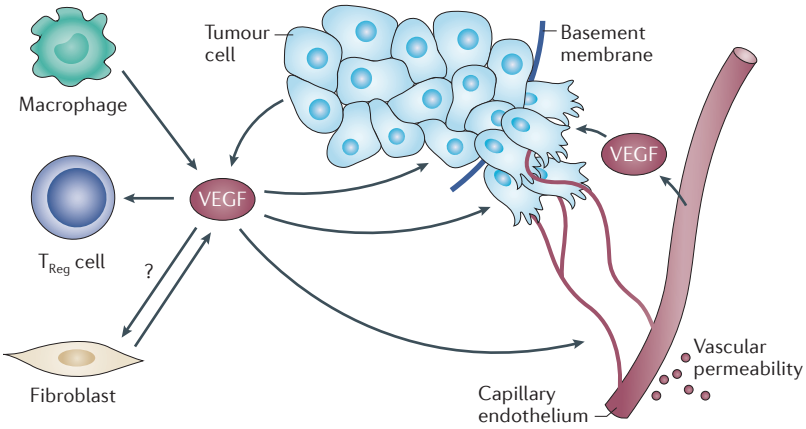

Figure 1 | **VEGF functions in tumours.** Vascular endothelial growth factor (VEGF) that is secreted by tumour and stromal cells, including macrophages, endothelial cells and fibroblasts, has multiple functions in the tumour microenvironment, which involve the ability of VEGF to interact with VEGF receptors that are expressed on different cell types. VEGF functions as a primary stimulus for angiogenesis, which is a process that involves the ability of VEGF receptors to stimulate signalling pathways that induce the proliferation and the migration of endothelial cells, and the ability of these cells to degrade and to remodel the extracellular matrix. These processes culminate in sprouting angiogenesis and the formation of new blood vessels. VEGF can also increase vascular permeability, which results in the deposition of a provisional fibrin matrix that triggers the formation of desmoplastic stroma. By contrast, VEGF secreted by tumour cells functions in an autocrine manner and promotes dedifferentiation and an epithelial–mesenchymal transition phenotype, with a consequent enhancement of tumour invasion and survival, and it can facilitate the function of cancer stem cells (FIG. 3). VEGF can also function as a chemoattractant to recruit regulatory T (T_{Reg}) cells that inhibit an antitumour immune response. Tumour fibroblasts also secrete VEGF. Neuropilin 1 that is expressed on tumour fibroblasts may contribute to tumour growth by nucleating fibronectin fibril formation, but it is not known whether this process involves VEGF. Arrows indicate the source and the targets of VEGF in tumours.

a causal link between tumour dedifferentiation and the activation of autocrine VEGF signalling, but more investigation is needed (see below). This hypothesis is also supported by the finding that expression of VEGF and VEGFR1 is induced by an epithelial–mesenchymal transition (EMT) of colon carcinoma cells[18] — a process that promotes dedifferentiation and progression to more aggressive tumours. Furthermore, VEGF stimulation of normal epithelial cells and differentiated carcinoma cells can induce an EMT[46,48].

However, the mechanism by which the signalling pathways that are associated with oncogenic transformation and dedifferentiation regulate the expression of VEGF and VEGF receptors is still unknown. Given that hypoxia-inducible factor (HIF)-mediated transcription is a major driver of VEGF expression in tumours, it is likely that hypoxia helps to establish autocrine signalling networks in tumour cells. An important observation is that aggressive tumour cells sustain HIF-mediated transcription[49], and mechanisms that have been implicated in inducing VEGF expression, such as RAS transformation[41,50] or EMT[18,46], probably directly affect HIF expression or activation. For example, the loss of oestrogen receptor-β (ERβ) expression that occurs in poorly differentiated prostate cancer and that causes an EMT phenotype, stimulates *VEGFA* transcription in tumour cells by a mechanism that involves transcriptional repression of prolyl hydroxylase 2 (PHD2; also known as EGLN1), which is an enzyme that targets HIF1α for degradation[51].

Several recent studies have provided insights into the mechanism of NRP regulation in cancer. Notably, Hedgehog signalling can induce NRP expression[38,52], which may be part of a positive feedback loop because NRP-mediated VEGF signalling can also induce the expression of the Hedgehog target gene GLI family zinc finger 1 (*GLI1*)[38,53]. The loss of PTEN induces *NRP2* transcription in prostate cancer through a mechanism that involves the JUN N-terminal kinase (JNK)–JUN pathway, which provides a direct link between the loss of a tumour suppressor and the induction of *NRP2* transcription[15]. Interestingly, both JUN and GLI1 can bind to the *NRP2* promoter and may function together to regulate *NRP2* transcription[15,38]. Expression of the transcription factor COUPTF2 (encoded by *NR2F2*) correlates with disease recurrence and progression in prostate cancer, and it can directly stimulate the transcription of *NRP2* (REFS 54,55). COUPTF2 can also suppress Notch signalling[56], which is interesting because there are reports that a Notch ligand — Delta-like 4 (DLL4) — can repress VEGFR2 and NRP1 expression[57], but another ligand (DLL1) can stimulate their expression[58]. Although more work is needed to understand how signalling pathways that contribute to tumour initiation and dedifferentiation regulate the components of VEGF signalling in tumour cells, the fundamental principle of this regulation has been established.

Autocrine VEGF signalling in tumour cells can also be regulated at the level of receptor trafficking, which enables intracellular VEGFR signalling (FIG. 2). Specifically, autocrine NRP1–VEGFR2 signalling in gliomas involves active VEGFR2 that is localized in a cytoplasmic compartment[14]. This finding is indicative of an increasingly popular view that intracellular VEGFR signalling is important[59] and that a key function of NRPs may be to promote the trafficking of VEGFRs and possibly of other growth factor receptors[60]. This mode of regulation has important implications for therapy (see below).

Functional interactions between VEGF receptors and other receptors. An emerging theme in the literature is that VEGF receptors interact with and affect the function of other growth factor receptors, which is a manifestation of growth factor receptor crosstalk (FIG. 2). In addition to the regulation of VEGF RTKs by NRPs, there are numerous reports that VEGF RTKs and NRPs interact with other growth factor receptors. VEGFR2, for example, forms a complex with MET — the receptor for hepatocyte growth factor (HGF) — in response to VEGF stimulation of glioblastoma cells, and VEGFR2 thereby regulates MET signalling[61]. As co-receptors, the NRPs are promiscuous and have numerous interactions with other receptors. NRP1 interacts with the MET receptor and enhances the ability of HGF to stimulate the invasion of pancreatic carcinoma cells[62] as well as the proliferation and survival of gliomas[63]. A direct interaction between the extracellular domain of NRP1 and the epidermal growth factor receptor (EGFR) has

Epithelial–mesenchymal transition
(EMT). A conversion from an epithelial to a mesenchymal phenotype, which is a normal component of embryonic development. In carcinomas, this transformation results in altered cell morphology, the expression of mesenchymal proteins and increased invasiveness.

Hypoxia-inducible factor
(HIF). A dimeric transcription factor that is formed of α- and β-subunits and that is involved in the hypoxia-sensitive regulation of numerous genes, including glycolytic enzymes, glucose transporters and angiogenic factors.

Table 1 | **Expression of VEGFs and VEGF receptors in human cancers***

VEGF or receptor	Cancer
VEGFs	
VEGF	Bladder[129,130], brain[14,131,132], breast‡ (REFS 38,133–135), colon‡ (REFS 87,136), gastric‡ (REF. 137), oral squamous‡ (REFS 138,139), lung‡ (REFS 140–143), mesothelioma‡ (REF. 144), myeloid leukaemia[145], ovarian[146,147], pancreatic[91,148] and prostate§ (REFS 149–151)
VEGFB	Breast‖ (REFS 134,152) and lung[140]
VEGFC	Breast[153], cervical‖ (REFS 153–155), colon‡‖ (REFS 153,156,157), gastric[158], oral squamous[159], lung‡ (REFS 140,153,160) and prostate[153]
VEGFD	Cervical[154], gastric[161] and lung[140]
PLGF	Breast¶ (REF. 162), colon‡ (REF. 136), gastric‡§‖ (REF. 163) and hepatocellular¶ (REF. 164)
VEGF receptors	
VEGFR1	Bladder[130], brain[131,132], breast‡ (REFS 133–135,152,165), colon[18,92], head and neck[166], lung‡ (REFS 140–142), melanoma[167], mesothelioma[144], myeloid leukaemia[145], oesophageal[168], ovarian[86,146,147], pancreatic (REFS 91,148) and prostate (REF. 169)
VEGFR2	Bladder§ (REF. 129), brain[14,131,132,161,170,171], breast‡ (REFS 133,135,172), cervical[173], colon§‖ (REFS 87,174), endometrial‡ (REF. 175), gastric[137], head and neck[166,176], hepatocellular‡ (REF. 177), lung‡ (REFS 140–142,178), melanoma[167], mesothelioma[144], multiple myeloma[179], myeloid leukaemia[145], oesophageal[168], ovarian[86,146,147], pancreatic[91,148], prostate[149,169], renal cell carcinoma[180], squamous[181] and thyroid‖ (REF. 182)
VEGFR3	Breast[153], cervical§ (REFS 153,154), colon‡‖ (REFS 153,156), gastric‡‖ (REFS 158,161), head and neck[159,166], lung‡§ (REFS 140,153,160), oesophageal[168] and prostate[153]
NRP1	Brain‖ (REFS 14,63,183,184), breast‡ (REFS 135,185,186), colon§ (REFS 83,187,188), lung[143,185,189], melanoma[167], ovarian[147,190,191], pancreatic[84,188,192–194] and prostate§¶ (REFS 150,151,195)
NRP2	Bladder[196], breast[38,186,197,198], colon[113,197], lung[143,189,197], melanoma[197,199], ovarian[190], pancreatic[193,194], prostate‡§ (REF. 15) and renal cell§‖ (REF. 200)

NRP, neuropilin; PLGF, placental growth factor; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor. *The data reported are primarily based on immunohistochemical analyses of tumours and indicate expression of VEGFs or VEGF receptors specifically in tumour cells. ‡Studies that showed a correlation between expression and poor survival or outcome. §Studies that showed a correlation between expression and disease stage or progression. ‖Studies that showed a correlation between expression and metastasis. ¶Studies that showed a correlation between expression and recurrence.

been shown, which augments the response of tumour cells to EGF and transforming growth factor-α (TGFα)[64]. This mechanism may contribute to the sustained activation of EGFR that occurs in some advanced cancers. Both NRP1 and NRP2 can interact with TGFβ receptors and can potentiate TGFβ signalling[65,66]. This finding has implications for EMT, which can be induced by either TGFβ signalling or by NRP-mediated VEGF signalling[46,65]. There is also evidence that NRPs can bind to specific growth factors in addition to VEGF and PLGF[67], including HGF[68], basic fibroblast growth factor (bFGF; also known as FGF2)[68], TGFβ[69] and platelet-derived growth factor (PDGF)[70]. However, whether this binding involves VEGF or whether it initiates a signalling response remains to be determined.

VEGF receptors also interact with specific integrins and activate or enhance integrin signalling in tumour cells (FIG. 2). This concept was established using the observation that VEGF signalling that is mediated by VEGFR2 can activate the ligand-binding function of multiple integrins in both endothelial cells and tumour cells by a mechanism that involves the PI3K–AKT pathway[71]. This mode of regulation may be bidirectional because there is also evidence that the αvβ3 integrin can form a complex with VEGFR2 and can increase the level of phosphorylation of this RTK in response to VEGF[72]. NRPs can also associate with specific integrins and can enhance their function in tumour cells[73,74]. A salient example of this interaction occurs between VEGF-bound NRP2 and the α6β1 integrin in breast carcinoma cells. NRP2, but not NRP1, co-immunopurifies with this integrin, and NRP2-mediated VEGF signalling enables α6β1 integrin to bind to its matrix ligand laminin, to engage filamentous actin, to form focal adhesions and to activate focal adhesion kinase (FAK)[74]. Interestingly, NRP2 is localized in focal adhesions that form on laminin, and this observation provides a direct connection between VEGF signalling and focal adhesion dynamics and signalling; this connection is corroborated by data from endothelial cells[75,76].

Collectively, the data that are currently available highlight a crucial role for the NRPs in regulating growth factor receptor and integrin signalling (FIG. 2), and this aspect of NRP function may underlie the contribution of NRPs to tumour biology. However, much remains to be learnt about the mechanisms by which NRPs interact with growth factor receptors and integrins, and how they potentiate their function[77]; for example, does the ability of NRPs to co-immunopurify and to colocalize with RTKs and integrins reflect their association in macromolecular complexes that contain other signalling molecules and endocytic components? Such complexes could regulate and facilitate VEGF signalling and receptor trafficking. The presence of such complexes could be an explanation for most of the data

Focal adhesions
Dynamic, macromolecular protein complexes that link the extracellular matrix to the actin cytoskeleton through integrins.

Box 2 | **Semaphorins and plexins in tumour cells**

A discussion of vascular endothelial growth factor (VEGF) signalling in tumour cells must include a mention of semaphorins — especially class 3 semaphorins (SEMA3s) — because they are secreted by tumour cells, they function as neuropilin (NRP) ligands and they have been implicated in tumour-associated functions. A prevailing idea is that SEMA3s and VEGFs carry out antagonistic effects on tumour cells: SEMA3s inhibit tumour growth, migration and invasion, and, by contrast, the VEGFs have pro-tumorigenic functions[33,124]. This idea is substantiated by the recent report that triple-negative breast tumours, which are poorly differentiated tumours and which have a high frequency of cancer stem cells, are characterized by high VEGFA expression and low SEMA3 expression[125]. However, there are a few reports indicating that SEMA3s can have a pro-tumorigenic function[33,124]. SEMA3s function by binding to NRPs in a complex with plexins — primarily type A plexins. Plexins are the only transmembrane receptors that can directly interact with small GTPases[126]. SEMA3 binding to type A plexins triggers the collapse of the actin cytoskeleton, which results in impaired migration and invasion. Interestingly, other plexin family members such as plexin B1, which binds to SEMA4D, are upregulated or mutated in some cancers, such as prostate carcinomas[127]. In breast cancer, the ability of the receptor tyrosine kinase ERBB2 to activate the pro-invasive small GTPases RHOA and RHOC is mediated by plexin B1, and this effect is independent of NRPs[128]. Clearly, there is much more to be learnt about semaphorins and plexins in cancer, and their relationship to VEGF signalling. An important issue that should be investigated in more detail is the role of plexins in VEGF–NRP signalling.

on VEGF signalling in tumour cells, such as the regulation of RTK activity by NRPs and the role of NRPs in receptor trafficking.

Another timely issue is how NRPs mediate VEGF signalling independently of VEGF RTKs. Several studies have shown VEGF signalling in cells that lack detectable VEGF RTK expression or involvement; for example, NRP-mediated VEGF signalling was reported to promote the initiation of renal cell carcinoma in the absence of detectable VEGF RTK expression[16]. In addition, the ability of NRP2-mediated VEGF signalling to affect prostate cancer is not inhibited by bevacizumab, which blocks VEGF interactions with VEGFRs but not with NRPs[15]. A probable mechanism to explain these phenomena is that NRPs signal by affecting the function of other RTKs and integrins, as discussed above. However, NRPs may signal independently of RTKs (FIG. 2). Specifically, the cytoplasmic domains of NRP1 and NRP2 contain a PDZ-binding domain that can bind to PDZ-containing proteins, especially GIPC1 (also known as NRP-interacting protein (NIP)). GIPC1 is a cytoplasmic scaffolding protein that interacts with a wide range of receptors and that contributes to receptor trafficking and signal transduction[78,79], and it has been implicated in tumorigenesis[79]. A recent study concluded that the NRP1 PDZ-binding domain is required to mediate the PLGF-stimulated growth of medulloblastoma, and that this is independent of VEGFR1 activity[80]. The PDZ-binding domain is thought to function by forming scaffolding complexes that transduce NRP signals; this hypothesis is corroborated by the finding that GIPC1 mediates the interaction of NRP1 with ABL1, which is a tyrosine kinase that could mediate NRP1 signalling[11]. GIPC1 can also function as a 'bridge' to promote the association of receptors that contain PDZ-binding domains such as NRPs and integrins[81]. Interestingly, the NRP2 cytoplasmic domain contains a motif that has a partial consensus sequence to an immunoreceptor tyrosine-based activation motif (ITAM)[31], although there is no evidence yet that this motif is functional.

PDZ-binding domain
(PSD95, DLG and ZO1-binding domain). A structural, protein–protein interaction domain, which is ~80–90 amino acids in length, that often functions as a scaffold for signalling complexes and/or as a cytoskeletal anchor for transmembrane proteins.

Immunoreceptor tyrosine-based activation motif
(ITAM). A motif (YXXL or YXXI) that can be phosphorylated in response to receptor ligation and that functions as a docking site for other proteins involved in signal transduction.

Autophagy
A cellular response in which the cell metabolizes its own contents and organelles to maintain energy production. Although such a process can eventually result in cell death, it can also be used to maintain cell survival in conditions of limiting nutrients.

VEGF-mediated functions in tumour cells

VEGF regulates key functions of established tumour cells. The overarching theme in this Review is that VEGF signalling in tumour cells markedly affects tumour function and that this is independent of VEGF-mediated angiogenesis and vascular permeability. The initial reports that described the effects of VEGF on tumour cells showed that autocrine VEGF signalling — particularly signalling that is mediated by VEGF RTKs and NRPs — can promote the growth, survival, migration and invasion of cancer cells[18,36,62,73,82–91]. Most of these studies implicated dominant signalling pathways (for example, the PI3K–AKT and MAPK pathways) as the mechanism by which VEGF influences these processes; for example, VEGFR1 promotes the migration and the invasion of colorectal carcinoma cells by stimulating the activation of ERK1 or ERK2 as well as the activation of JNK and the consequent translocation of the p65 (also known as RELA) subunit of nuclear factor-κB (NF-κB) into the nucleus[92]. VEGFR1 can also sustain the survival of colorectal carcinoma cells that have undergone an EMT[18]. Several studies have described the ability of NRP-mediated VEGF signalling to affect the survival of tumour cells by activating the PI3K–AKT pathway; for example, NRP1-mediated VEGF signalling is able to sustain the survival of breast carcinoma cells[36,82].

Recent studies have shown that the role of VEGF signalling in tumours might be more complex than initially thought; for example, the above-mentioned VEGF-induced VEGFR2–MET complex in glioblastoma cells contains a tyrosine phosphatase, which inhibits HGF-mediated invasion and mesenchymal transition[61]. These findings need to be reconciled with other reports that implicate VEGF and VEGFR2 in the function of glioma stem cells[14] (see below). In addition, it has been reported that NRP1 on tumour myofibroblasts nucleates fibronectin fibril assembly by a mechanism that involves the α5β1 integrin and that NRP-mediated fibril assembly contributes to tumour growth[11], which improves our understanding of the importance of NRP signalling in the tumour microenvironment. However, this study did not investigate the contribution of specific NRP ligands and it is therefore not known if the mechanism is VEGF dependent. VEGF may also regulate autophagy because it has been shown that NRP2-mediated VEGFC signalling mediated by mTOR complex 1 activates an autophagic mechanism that combats chemotherapy-induced stress, which has implications for the role of VEGF signalling in therapy resistance[93].

VEGF, cancer stem cells and tumour formation. The importance of VEGF and VEGF receptor functions in cancer has been highlighted by the recent reports that autocrine VEGF signalling has a causal role in tumour formation and in the function of cancer stem cells, and these reports have distinguished this growth factor from many others (FIG. 3). Despite some controversy

CD133
A cell-surface glycoprotein, which is also known as Prominin 1, that can be used as a marker for some cancer stem cells.

regarding the existence and the nature of such cells, it is evident that many tumours harbour a small population of cells that have self-renewal potential and the ability to initiate the growth of new tumours[94]. An initial study using a transgenic mouse model showed that autocrine VEGF signalling synergizes with EGFR signalling to promote the development of squamous carcinoma[41]. Importantly, this study concluded that the effect of VEGF is cell autonomous and angiogenesis independent. Mechanistically, VEGF was shown to mediate an autocrine proliferation loop that involves VEGFR1 and NRP1. A rigorous analysis of the early stages of squamous carcinoma formation in the skin resulted in several seminal findings that suggest that autocrine VEGF signalling is directly involved in the function of cancer stem cells[40]. In early-stage tumours or papillomas, cancer stem cells are localized in a perivascular niche that occurs adjacent to endothelial cells. Blocking VEGFR2 reduced the size of the cancer stem cell pool and their self-renewal potential. Conditional deletion of *VEGFA* in the tumour cells of established tumours caused tumour regression by decreasing both microvascular density and the proliferation and renewal of cancer stem cells. Moreover, genetic deletion of *NRP1* prevented the ability of VEGF to promote stem cell-like properties and self-renewal. These findings, which were observed both *in vivo* and *in vitro*, clearly establish the importance of autocrine VEGFA signalling that is mediated by NRP1 and VEGFR2 in cancer stem cells. The localization of cancer stem cells adjacent to endothelial cells infers that tumour cell-derived VEGFA functions both as a paracrine factor to stimulate angiogenesis and as an autocrine factor to regulate cancer stem cells (FIG. 3). However, the localization of cancer stem cells adjacent to the endothelium needs to be reconciled with other reports showing that hypoxia drives the self-renewal of cancer stem cells[95].

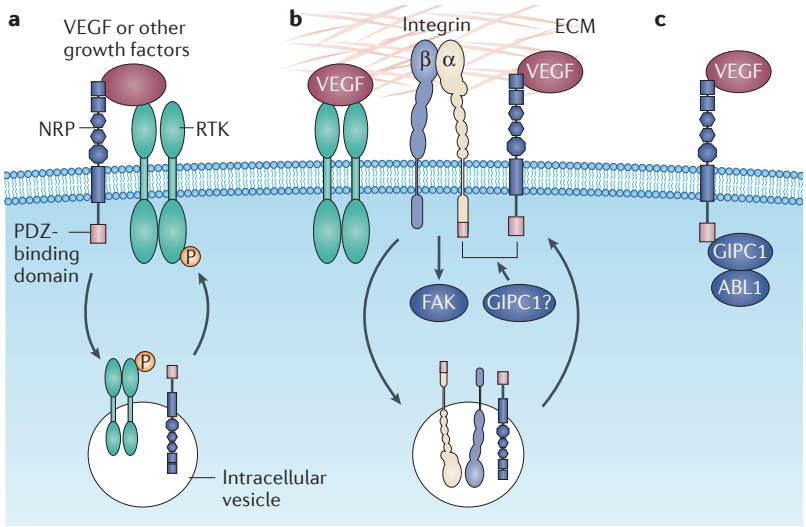

Figure 2 | **Receptor interactions that promote VEGF signalling in tumour cells, and the central role of NRPs.** **a** | Neuropilins (NRPs) interact with and potentiate the signalling function of growth factor receptor tyrosine kinases (RTKs), including vascular endothelial growth factor (VEGF) RTKs. This mode of regulation may be associated with internalization of the RTK and signalling from an intracellular compartment. Several growth factors, including hepatocyte growth factor, basic fibroblast growth factor, platelet-derived growth factor and transforming growth factor-β, directly interact with NRPs, but whether this binding is sufficient by itself to induce a signalling response is not known. **b** | NRPs also interact with specific integrins and activate their ability to bind to extracellular matrix (ECM) ligands, which results in the stimulation of integrin-mediated signalling through focal adhesion kinase (FAK). The RTK VEGF receptor 2 (VEGFR2) can also function in a similar capacity. In addition, NRPs may regulate integrin function by promoting their endocytic recycling. Both NRPs and specific integrin α-subunits (α5 and α6) contain a PDZ (PSD95, DLG and ZO1)-binding domain (Ser-Glu-Ala) at their carboxyl terminus, and PDZ proteins, such as the neuropilin-interacting protein GIPC1 might promote the association of these two classes of receptors. **c** | NRPs may also signal independently of other receptors, possibly by using their PDZ-binding domains to associate with signalling molecules such as ABL1. Note that these proposed mechanisms are not mutually exclusive, and there is the possibility that VEGF signalling in tumour cells involves the formation of macromolecular complexes that integrate components of these mechanisms.

An interesting theme that emerges from the above-described studies and other studies is that distinct VEGF family members and VEGF receptors can be used to facilitate tumour initiation and growth; for example, the two studies of squamous carcinoma formation that are discussed above implicated different VEGF RTKs. An explanation for this may be that the initial study identified a VEGFR1-mediated proliferation loop that contributes to tumour growth[41] and the second study suggested that VEGFR2 is directly involved in the function of cancer stem cells and their self-renewal[40]. It is worth noting that VEGFR1 has yet to be implicated in the function of cancer stem cells or in tumour initiation. Other studies have suggested that the contribution of autocrine VEGF signalling to tumour initiation is independent of VEGF RTKs and is driven by NRP signalling. Such a mechanism has been proposed for renal cell carcinoma[16]. In addition, a PLGF–NRP1 signalling axis that is independent of VEGFR1 contributes to the growth and to the spread of medulloblastomas[80]. Although it is possible that NRP signalling alone mediates autocrine VEGF signalling in this context, a more probable scenario (discussed above) is that NRPs potentiate the function of other non-VEGF receptors that are crucial for the function of cancer stem cells and tumour initiation. This idea is exemplified by our work on the role of VEGF–NRP2 signalling in the initiation of breast cancer. As mentioned above, VEGF–NRP2 signalling can activate the α6β1 integrin[38,74], which is noteworthy because this integrin is necessary for the function of some cancer stem cells[96,97]. Another important aspect of autocrine VEGF signalling in cancer stem cells is that this signalling can occur in an intracellular compartment. VEGFR2 and NRP1 are preferentially expressed on glioma stem cells that are positive for CD133, and ablation of either VEGFR2 or NRP1 in glioma cells *in vivo* increases apoptosis and reduces tumour formation[14]. Importantly, VEGF signalling that is mediated by NRP1 and VEGFR2 maintains a cytosolic pool of active VEGFR2 that may be the source of cell survival signalling and that is resistant to VEGF-specific antibody (bevacizumab) therapy.

Although there are data that clearly implicate VEGF in the function of cancer stem cells and tumour initiation, much less is known about the signalling mechanisms responsible for this function. An example of such a mechanism derives from our work on VEGF–NRP2-mediated activation of the α6β1 integrin. This activation induces activation of the FAK–RAS signalling pathway that culminates in non-canonical Hedgehog signalling,

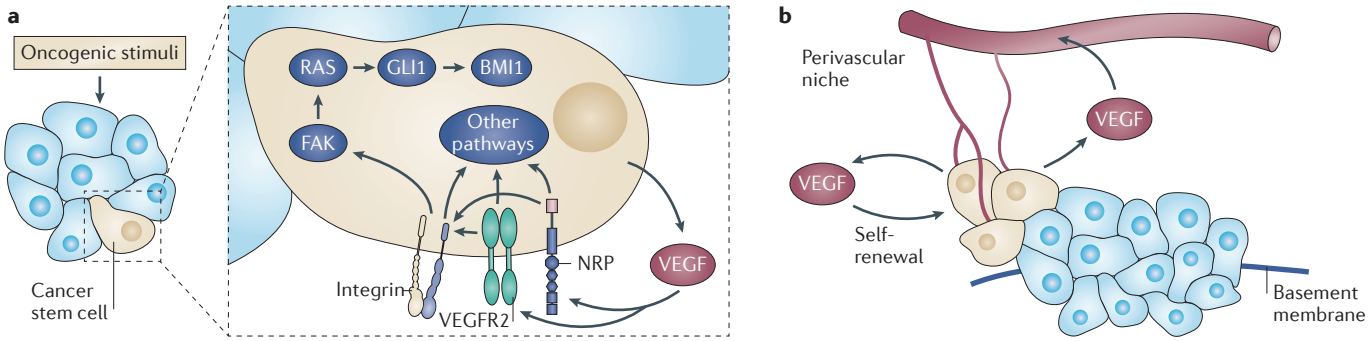

Figure 3 | **Role of autocrine VEGF signalling in the function of cancer stem cells and tumour formation.** **a** | The expression of vascular endothelial growth factor (VEGF) and VEGF receptors is induced concomitantly with oncogenic transformation; this facilitates the establishment of autocrine VEGF signalling. This signalling, which is mediated by the receptor tyrosine kinase VEGF receptor 2 (VEGFR2) and by neuropilins (NRPs), could be necessary for the function of cancer stem cells (beige cells) because it seems to maintain the size of the stem cell pool and to sustain self-renewal. The ability of autocrine VEGF signalling that is mediated by NRPs and integrins to regulate the expression of the Hedgehog target GLI family zinc finger 1 (GLI1) and the Polycomb group repressor BMI1 provides one mechanism to account for the contribution of autocrine VEGF signalling to the function of cancer stem cells, but other mechanisms probably exist. **b** | Cancer stem cells can be localized in a perivascular niche, which enables VEGF that is secreted by these cells to function in a paracrine manner to stimulate angiogenesis in nascent tumours. Autocrine VEGF signalling can also promote dedifferentiation and an epithelial–mesenchymal transition (EMT) phenotype that results in increased migration and invasion into the stroma. FAK, focal adhesion kinase.

which in turn activates GLI1. GLI1 then induces the expression of BMI1 (REF. 38) (FIG. 3), which is a Polycomb group transcriptional repressor that has been implicated in self-renewal and tumour initiation[98,99]. As mentioned above, GLI1 can stimulate the transcription of *NRP2* (REFS 38,52), which thereby creates a positive feedback loop that has the potential to sustain the self-renewal properties of cancer stem cells. Hedgehog signalling also has a crucial role in tumour cell–stromal cell interactions in this context, and this is shown by the finding that tumour-derived sonic hedgehog stimulates PLGF expression in stromal cells, which promotes the growth of medulloblastomas[80].

However, a fundamental issue is whether VEGF signalling alone can cause oncogenic transformation. Although there is a report that VEGFR1 can cause transformation[100], a more feasible hypothesis is that autocrine VEGF signalling is induced at the same time as other oncogenic events that drive tumour initiation, but that it can be an important (if not essential) component of the initiation process, as described above. In this context, a thought-provoking finding is that chronic inflammation causes upregulation of VEGFR2 in intestinal epithelial cells and that VEGFR2 signalling in these cells is required for tumour growth; this finding provides a causal link between inflammation and cancer that involves VEGF signalling[13]. However, this discussion of inflammation focuses on the role that autocrine VEGF signalling has in maintaining the function of cancer stem cells. Moreover, the data that are currently available clearly implicate autocrine VEGF signalling in sustaining self-renewal[38,40], which is consistent with the more general hypothesis that autocrine signalling is required to maintain a stem cell state[101]. Autocrine VEGF signalling is also closely associated with tumour dedifferentiation and with EMT[46], which are processes that may be involved in the genesis of cancer stem cells[101,102]. Moreover, autocrine VEGF signalling has been implicated in the metastatic cascade[36,80], and this is consistent with the recently identified role of cancer stem cells in tumour dissemination[102].

Although other growth factors may be involved in the autocrine signalling pathways that contribute to the function of cancer stem cells, VEGF is increasingly becoming recognized as a crucial factor. A potential explanation for this phenomenon is that the mechanisms that regulate VEGF expression — especially HIF-mediated transcription — are essential components of a stem cell phenotype[95,103]. The argument can be made that autocrine VEGF expression is a manifestation of HIF activation that is associated with the genesis of cancer stem cells and that is characteristic of poorly differentiated tumours[49,95]. For example, in high-grade prostate carcinoma, VEGF, HIF1α and NRP expression is higher in poorly differentiated tumour cells compared with more differentiated tumour cells[46].

Therapy

VEGF-targeted therapy — either alone or in combination with chemotherapy — is used for the treatment of many cancers[8]. Antibody-mediated inhibition of VEGF using bevacizumab is currently the predominant mode of VEGF-targeted therapy, although drugs that inhibit VEGF RTK activity (such as sunitinib and sorafenib) are also used[8]. The prevailing idea is that such therapy targets angiogenesis and other endothelial cell functions, and this aspect of VEGF-targeted therapy has been extensively studied and reviewed[8,104,105]. In this Review, we are interested in the possibility that targeting VEGF and VEGF receptors specifically on tumour cells could be effective in light of our increasing understanding of the importance of autocrine VEGF signalling in tumour initiation and in the biology of aggressive cancers. However, a

Polycomb group
Proteins that were first described in *Drosophila melanogaster* and that are required for normal development. They work in multiprotein complexes that are called Polycomb repressive complexes, which establish regions of chromatin in which gene expression is repressed.

potential caveat could be that although VEGF-targeted therapy (primarily bevacizumab) has reduced tumour burden and improved survival in some cancers, it has not been as successful as initially anticipated[106]. If we assume that bevacizumab has the potential to inhibit autocrine VEGF signalling in tumour cells, the modest effect of this drug that has been observed so far would diminish the importance and therapeutic potential of targeting VEGF signalling in tumour cells. However, a crucial observation is that bevacizumab does not inhibit the interaction of VEGF with NRPs[107]. Given the importance of NRPs to cancer stem cells and to VEGF signalling in tumour cells, which has been established in preclinical studies, this observation has widespread therapeutic implications and indicates that therapies that target NRPs or VEGF–NRP interactions could be very effective, especially when they are used in combination with antibodies against VEGF[108] (FIG. 4). Interestingly, clinical trials involving patients with advanced gastric and breast cancer assessed the efficacy of bevacizumab and concluded that high NRP1 expression is prognostic of a poor response to bevacizumab[109,110], reinforcing the importance of targeting NRP and the need for combination therapy.

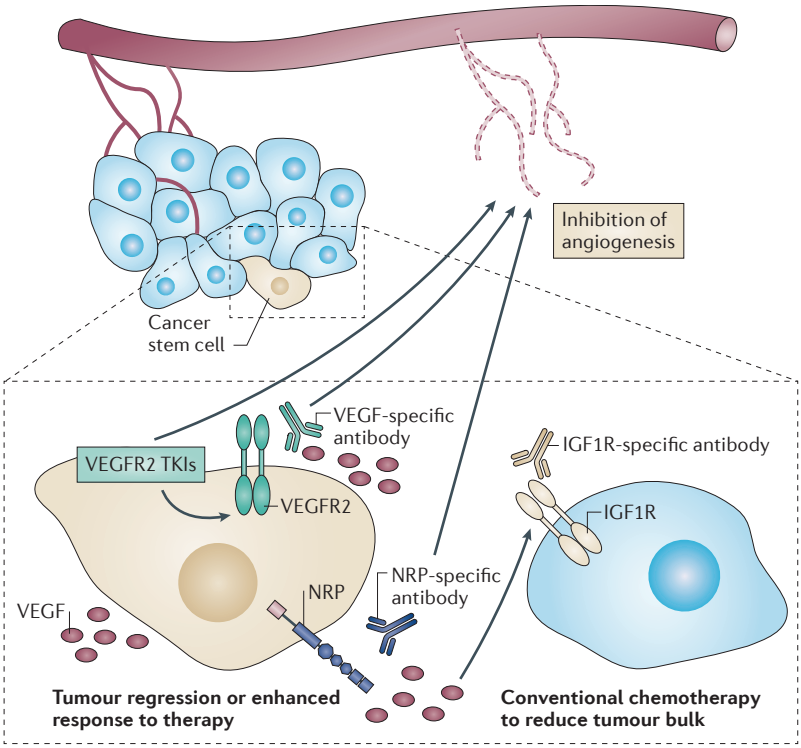

Figure 4 | **Therapeutic targeting of VEGF signalling in tumour cells.** The functional importance of vascular endothelial growth factors (VEGFs) and VEGF receptors — that is, neuropilins (NRPs) and VEGF receptor tyrosine kinases (RTKs) — that are expressed by tumour cells, in particular those that are expressed by cancer stem cells (beige cells), provides an important opportunity for the development of new therapeutic approaches, especially for highly aggressive tumours. These approaches have the potential to promote tumour regression and to improve the response to standard chemotherapy and radiation therapy. So far, strategies that inhibit VEGF signalling have primarily focused on targeting angiogenesis using either bevacizumab to inhibit VEGF or tyrosine kinase inhibitors (TKIs) that target VEGF RTKs such as VEGF receptor 2 (VEGFR2). NRPs are becoming recognized as crucial effectors of autocrine VEGF signalling in tumours, and more emphasis should be placed on targeting them therapeutically. Although some side effects were observed during the initial clinical use of a humanized NRP1 antibody, targeting NRPs is still a potentially effective strategy, and approaches need to be developed that minimize these side effects. It is also important to note that bevacizumab does not inhibit the binding of VEGF to NRPs, which highlights the importance of targeting NRPs directly and developing VEGF-specific reagents (such as placental growth factor (PLGF)-specific antibodies) that inhibit NRP binding. Targeting NRPs can result in compensatory signalling by other growth factor receptors, which indicates the potential importance of using a combination therapy. This possibility is shown by the compensatory insulin-like growth factor 1 receptor (IGF1R) signalling that occurs in prostate cancer, and it is likely that other mechanisms of compensation in response to VEGF pathway inhibition will be discovered for other tumour cells. These approaches may benefit from the use of conventional chemotherapy to reduce overall tumour burden.

Targeting NRPs and VEGF RTKs. Preclinical data suggests that targeting NRPs could potentially be used as a mode of cancer therapy. NRP expression is minimal in most adult tissues, which reduces the possibility that NRP-based therapies would perturb normal tissue function, and mouse studies using therapeutic NRP-specific antibodies have reported minimal side effects[108,111]. In addition, function-blocking NRP1- and NRP2-specific antibodies have been shown to inhibit tumour growth and to cause stasis in mice[38,80,108,112]. Similar results have been achieved using RNA interference (RNAi) to deplete NRP expression[113]. Other therapeutic approaches include the use of small peptides that prevent NRP oligomerization[114] or soluble forms of NRPs that function as decoy receptors[107]. Importantly, most of these studies concluded that targeting NRPs had a direct effect on tumour cells *in vivo*, and some studies showed that there was little effect on tumour angiogenesis[38].

Unfortunately, recent Phase I results using MNRP1685A, which is a humanized NRP1-specific monoclonal antibody, have raised concerns about targeting NRPs for therapy. MNRP1685A is cleared from the circulation more rapidly than other humanized antibodies, which suggests that it may have off-target effects[115]. Another concern is the preliminary report that patients who were dosed with MNRP1685A in combination with bevacizumab showed frequent but transient decreases in platelet levels and clinically significant proteinuria[116]. Given that NRP1 can affect the function of multiple growth factor receptors, these effects of MNRP1685A may not be surprising. However, these results should not discourage future work on the therapeutic targeting of NRPs, given the biological importance of NRPs in cancer. Strategies that target NRPs on tumour cells or cancer stem cells more specifically may decrease potential toxicity and off-target effects. The discovery that peptides with a carboxy-terminal arginine residue bind to NRP1 and NRP2 on the cell surface has been exploited as a novel approach to deliver cytotoxic peptides to tumour cells, and such tumour-penetrating peptides can be used to facilitate the delivery of co-administered drugs directly to tumour tissue[109,117]. Another novel NRP-targeting method that has important implications for therapy comes from our work that showed that the inhibition of NRP2 in prostate cancer cells induces the expression of the insulin-like growth factor 1 receptor (IGF1R) and triggers downstream signalling, which increases tumour proliferation[15]. However, NRP2 and IGF1R combination

therapy proved to be effective in reducing tumour burden (FIG. 4). This study also found that NRP2 is a valid biomarker for predicting the response to IGF1R therapy.

Some studies have investigated the effect of blocking VEGF RTKs or their activity in tumour cells[14,40,118,119]. Of note, antibody-mediated inhibition of VEGFR2 in the mouse model of skin cancer that is discussed above caused tumour regression by reducing the cancer stem cell pool size and by impairing cancer stem cell renewal, as well as by decreasing microvascular density[40]. In addition, the inhibition of VEGFR2 expression or activity blocked the VEGF–VEGFR2–NRP1 signalling axis. This inhibition also impeded the viability of glioma stem cells *in vitro* and increased the survival of mice that harboured glioma xenografts[14]. The interesting result of this study, which has been alluded to above, is that VEGFR2 signalling occurs intracellularly and is blocked by inhibitors of VEGFR2 tyrosine kinase activity but not by bevacizumab. In this context, it is thought-provoking that the inhibition of VEGFR2 expression in ovarian carcinoma cells has been shown to result in increased tumour growth *in vivo* and was associated with increased VEGF and NRP1 expression[119]. This finding confirms the importance of NRPs in tumour cells[119].

Targeting VEGF. Despite the concerns that relate to bevacizumab, the targeting of VEGF family members has the potential to be a very effective approach for inhibiting tumour cell function. This is supported by the report that bevacizumab treatment of patients with locally advanced breast cancer significantly increased tumour cell apoptosis[120]. In addition, antibody-mediated inhibition of PLGF in medulloblastomas had a direct antitumour effect *in vivo* and caused tumour regression[80], and it had minimal side effects. Similar results were achieved by blocking NRP1, but VEGFR1 inhibition had no effect. These findings substantiate the feasibility of using antibodies that are specific for other VEGF family members that block binding to NRPs. The potential of targeting VEGF is validated by the suppression of pancreatic carcinoma cell tumorigenesis by using a 'VEGF-trap' that sequesters VEGF[121].

The above findings are tempered by the report that anti-angiogenic therapy involving inhibition of either VEGF or VEGF RTKs increased tumour invasion and metastasis[122]. Of particular relevance, conditional deletion of *VEGFA* in pancreatic carcinoma cells, which should disrupt autocrine VEGF signalling, increased tumour invasiveness. By contrast, conditional deletion of *VEGFA* in established squamous carcinomas caused tumour regression[40]. An explanation for these seemingly contradictory findings is that *VEGFA* deletion inhibits slow-cycling cancer stem cells and selects for cells that proliferate at a higher rate and that may — at least in the short-term — be invasive. This hypothesis is supported by the above-mentioned finding that NRP2 inhibition in prostate cancer, which targets a putative stem cell population, increases IGF1R-mediated cell proliferation[15]. Although more work is needed to understand these fundamental issues, it is becoming evident that combined modes of therapy will be necessary to target VEGF signalling in tumour cells.

Conclusions and future perspectives

The salient theme of this Review is that VEGF signalling in tumour cells — especially autocrine signalling — can be an essential component of tumour initiation and it can be intimately associated with oncogenic transformation. More specifically, compelling data indicate that VEGF signalling promotes the function of cancer stem cells and sustains their self-renewal. These functions of VEGF are independent of its contribution to angiogenesis and, for this reason, constitute a paradigm shift in our understanding of the role of VEGF in cancer. Continued work to understand the relationship between autocrine VEGF signalling and the biology of cancer stem cells is warranted; in particular, because of the potential of using VEGF signalling as a therapeutic target.

VEGF signalling in tumour cells can involve VEGF RTKs, other RTKs, NRPs and integrins, but NRPs seem to be at the centre of signalling events that enable VEGF to affect tumour cell function, especially tumour initiation and the function of cancer stem cells. Much remains to be learnt about how NRPs function in this context, but it is evident that they have the ability to regulate the function and the trafficking of RTKs and integrins. An important issue in the future will be determining the extent to which NRP regulation of other receptors involves VEGF. Moreover, how this regulation occurs is only beginning to be understood, and the possibility that NRPs are components of macromolecular signalling complexes merits particular attention. NRPs may also have some semi-autonomous signalling potential that derives from their ability to interact with PDZ domain-containing proteins. Both NRP1 and NRP2 have been implicated in the function of cancer stem cells and are thought to have other functions in tumours, but the relative contributions of these two receptors is not yet known. Related issues are whether the NRP2 cytoplasmic domain variants have functional differences and the extent to which NRP glycosylation affects their ability to promote tumour initiation. It is somewhat surprising that the contribution of VEGF RTKs to VEGF signalling in tumour cells has not been consistent among studies — especially given the dominant role of VEGFR2 in driving angiogenesis — and it will be important to understand why their functional importance is diminished in some tumour cells.

The realization that autocrine VEGF signalling can be crucial for tumour initiation and for the characteristics of highly aggressive cancers provides a promising opportunity for the development of new therapeutic approaches. Such approaches are particularly intriguing because NRPs seem to be essential for this VEGF signalling and can be therapeutically targeted using currently available reagents. However, this excitement is tempered by the complexities that are associated with targeting VEGF and VEGF receptors, including potential toxicity, the possibility that cells resistant to such therapy can be highly aggressive and the possibility that compensatory signalling mechanisms may offset potential benefits. The development of more effective strategies will probably involve approaches that target tumour cells more specifically as well as the use of a combination of therapeutic reagents that overcome the resistance caused by targeting single molecules.

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